SPECIFICATION

NOVEL HEMOLYTIC ACTIVE PROTEINS AND GENES ENCODING THE SAME

TECHNICAL FIELD

The present invention relates to proteins having a hemolytic activity and genes encoding thereof. More specifically, the present invention relates to novel proteins having the hemolytic activity, a process for producing and the use of the same.

BACKGROUND ART

The sting injury by the jellyfish in sea bathing has occurred in various parts of the world. The sting injury by *Carybdea rastonii* or *Physalia physalis* has also occurred frequently in Japan every year in the season of sea bathing of the summertime. The degree of the symptom by sting differs by species of a jellyfish and the individual differences of patients. The first symptom is dermotoses, such as pain, flare, papule, vesicle and so on in the sting site. In a serious illness, patients may die with generating of hemorrhagic maculae and the necrosis, and also constitutional symptom, such as headache, high fever, nausea, dyspnea, and the fluctuation of a pulse. Although such sting injury is occurring frequently, the determination and pharmacological properties of the toxic components of jellyfish have not been studied intensively. Therefore, the development of medicines for treatment of the sting by the jellyfish is hardly performed before the present invention.

The studies on the toxic components of *Carybdea rastonii* have reported by Sato et al., and they ~~cleared~~ found that there are ~~same~~ some active substances having physiological activities, such as hemolysis, platelet agglutination, mast cell degranulation, the vessel smoothness muscle contraction, the dermal necrosis, the heart poison and the fatality in the crude extract fractions from the freeze-dried tentacle of *Carybdea rastonii.* They also examined on the platelet agglutination effect and vessel smoothness muscle contraction effect of the toxic component (Akihiko Sato, "Research on the toxic component of *Carybdea rastonii*", *The Journal of the Ochanomizu Medico-dental Society,* vol. 33, No. 2, 131-151, June, 1985).

On the one hand, since the poison from the nematocyst of a jellyfish was a non-dialyzable high polymer and deactivated by treatment with acid or alkali, or by heating processing, organic solvent processing, protease processing, etc., it was thought that the main components of this poison were proteins.

Moreover, the purification of the protein toxin derived from a jellyfish has also been tried; however, the isolation and the purification of the active components maintaining the hemolytic activity were not performed since the toxin of a jellyfish itself was very easy to be deactivated. Therefore, the physical and chemical properties of the toxin from jellyfish ~~have never been clarified up to now~~ were not known up to now.

The detailed studies on the toxic component of a jellyfish is very important for the development of drugs applying their various physiological activities, in particular, specific hemolytic activity and the platelet agglutination effect.

Therefore, the problems to be solved by the present invention is providing an approach to development of the drugs for treatment of the sting injury by the jellyfish by means of isolating the proteins or peptides having as potent hemolytic activity as possible, in the state where the physiologic activity is retained. The present invention further provides the approach to study similarities on embryology or structure, and the species specificity of the protein having hemolytic activity to evaluate the structure-activity relationship thereof.

DISCLOSURE OF THE INVENTION

The inventors extensively performed the research for isolating the proteins having the hemolytic activity from the nematocyst of *Carybdea rastonii* using the hemolytic activity as the parameter, while retaining these hemolytic activities. As the result, they found out the process for isolating and purifying the proteins retaining hemolytic activities, and ~~clarified~~ found that the protein from *Carybdea rastonii* ~~having~~ had the partial chemical structure consisting the following amino acid sequences (1)-(3), and the molecular weight of about 50,000 Da (determined by SDS gel electrophoresis).

Amino acid sequence (1):

Gly-Glu-Ile-Gln-Thr-Lys-Pro-Asp-Arg-Val-Gly-Gln-Ala-Thr (SEQ ID NO:1)

Amino acid sequence (2):

Gly-Asn-Ala-Glu-His-Val-Ala-Ser-Ala-Val-Glu-Asn-Ala-Asn-Arg-Val-Asn-Lys (SEQ ID NO:2)

Amino acid sequence (3):

Met-Ser-Asp-Gly-Phe-Tyr-Thr-Met-Glu-Asn-Ser-Asp-Arg-Arg-Lys (SEQ ID NO:3)

(wherein, an amino acid residue is written by the 3 letters notation defined by IUPAC and IUB)

Furthermore, they prepared the primers based on their partial chemical structures of the protein, and analyzed the gene sequence of about 1,000 base pair of said protein by conducting the RT-PCR ~~to~~ on the total RNA prepared from the tentacle of *Carybdea rastonii* by using these primers. Consequently, they further determined the full primary amino acid sequence of the hemolytic active protein of *Carybdea rastonii* by means of analyzing the gene sequence ~~in~~ at the 5'-end and 3'-end using the 5' RACE method and 3' RACE method.

Therefore, one embodiment of the present invention provides the specific protein having above-mentioned physiological, physical and chemical properties and represented by the amino acid ~~SEQ ID NO 5.~~ (SEQ ID NO:5) or the amino acid sequence thereof partially modified by the deletion or substitution of amino acid, and /or the amino acid sequence thereof partially modified by the deletion or substitution of amino acid further one or more amino acids are added.

Another embodiment of the present invention also provides the process for preparing such proteins.

Furthermore, another embodiment provides the gene encoding such proteins, the process for preparing the specific

proteins using the gene, and the drugs or the pesticides using the same.

The present invention further provides the pharmaceutical compositions or the pesticides containing the proteins using these properties, particularly, the pharmaceutical compositions having the platelet agglutination effect etc.

Moreover, since a specific antibody can also be obtained from this hemolytic active protein according to a conventional method (Cell Technology, separate volume, "*Experimental protocol of antipeptide antibody*", Shujunsha Co.), the present invention also provides the pharmaceutical compositions containing said antibody.

BEST MODE FOR CARRYING OUT THE INVENTION

The isolation and purification of the proteins having the specific physiological activity provided by the present invention can specifically be performed as follows. For example, the ultrasonication of the nematocyst of *Carybdea rastonii* is carried out in phosphoric acid buffer solution, and then supernatants are collected by the centrifugal separation to obtain a crude extract. The object proteins can be separated and purified by subjecting this crude extract to ion exchange high performance liquid chromatography using TSK-GEL (Toso Co.), and the gel filtration high performance liquid chromatography with Superdex-75 (Pharmacia Co.).

The structure of the protein provided according to the present invention obtained in this way can be determined by combining the analysis procedure of the amino acid sequence by the selective degradation using the enzyme, and the analysis

procedure of a gene sequence using the PCR method etc. For example, the amino acid sequence can be determined by processing the protein separated and purified as mentioned above with a lysylendopeptidase, fractionating the fragment using a high performance liquid chromatography, and analyzing it using an amino acid sequencer etc. Next, the gene sequence of the proteins can be determined by RT-PCR method etc. using the primers prepared on the basis of the amino acid sequence. Finally, the full primary amino acid sequence of the proteins can be clarified by determining the amino acid sequence on the basis of the gene sequence.

It was confirmed by such analysis that the protein provided according to the present invention has the molecular weight of about 50,000 Da (measured by SDS gel electrophoresis), and the partial amino acid sequences have the above-mentioned amino acid sequences (1) to (3).

As a result of homology search on the partial amino acid sequences, the homology between the protein of the present invention and the known proteins was very low. Therefore, it was suggested that the protein of the present invention having the hemolytic activity is completely novel protein, which is not similar to the known proteins.

Next, the determination of the gene sequence of about 1,000 base pairs by performing RT-PCR to total RNA prepared from the tentacle of *Carybdea rastonii* using the primers prepared on the basis of the partial amino acid sequence, and the determination of the gene sequences of the 5'-end and the 3'-end

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using the 5' RACE method and 3' RACE method were performed. Consequently, it is concluded that the hemolytic active protein of *Carybdea rastonii* has the full primary amino acid sequence represented by ~~SEQ ID NO 5~~ (SEQ ID NO:5), and the gene encoding thereof has the base sequence represented by ~~SEQ ID NO 4~~ (SEQ ID NO:4).

The result of the homology search on these full primary amino acid sequences exhibited that the homology between the protein and the known proteins was low.

The method for preparing the specific protein of the present invention by separation and purification is characterized in retaining the hemolytic activity. For example, the separation and the purification in the state of retaining such hemolytic activity are attained by performing the processing such as ultrasonication using the above-mentioned phosphoric acid buffer solution or various high performance liquid chromatography in 10 mM phosphoric acid buffer solution (pH 6.0) containing above 0.1 M NaCl, preferably above 0.3 M, and more preferably above 0.5 M, at below 10℃, preferably below 5℃.

Therefore, the present invention also provides the method for preparing the protein by extracting and purifying them from the nematocyst of the *Carybdea rastonii* in the state of retaining the physiological activity.

The specific protein of the present invention also can be prepared by the gene recombination method. Preparation by the gene recombination method can be performed according to a conventional method. For example, it can be obtained by preparing the vector integrated with the gene represented by

a (SEQ ID NO: 4)
~~SEQ ID NO 4~~, transforming a host cell by the vector, incubating or growing the host cell, and isolating and purifying the proteins having hemolytic activity of interest from the host cell or culture solution.

Since the protein provided according to the present invention has a hemolytic activity, for example, it may be used for the medicaments having the platelet agglutination effect and for the reagents for research on a hemolysis. Furthermore, it provides the new approach for the development of drugs, such as a drug for treating the sting by the jellyfish, and development of pesticides, such as an insecticide, using the hemolytic activity.

EXAMPLES

The present invention will be described in detail with reference to the following examples; however, the present invention is not limited to the examples.

Example 1

1) Extraction of the nematocyst of *Carybdea rastonii*

200 mg of the nematocyst of the *Carybdea rastonii* obtained on the Miura peninsula, Kanagawa, Japan and cryopreservated at -80℃ was immersed in 8 ml of 10 mM phosphoric acid buffer solution (pH 6.0), and treated for 15 minutes by the ultrasonic wave (ultrasonic cleaner VS150, Iuchi Co.). The supernatant fluids were collected by centrifugal separation (3,000rpm, for 20 minutes). This operation was performed 3 times in total. Furthermore, the same extraction operation was repeated 3 times with 8 ml of 10 mM phosphoric acid buffer solutions (pH 6.0)

containing 1 M NaCl, and then all the supernatant fluids were collected. After the extraction operation, ion exchange HPLC (high performance liquid chromatography) of the following purification step was immediately performed.

2) The purification by ion exchange HPLC (column: TSK-GEL CM650S, column size: 20 x 220 mm)

The above-mentioned column was equilibrated with 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.3 M NaCl. After the equilibration, the supernatant fluids obtained by extraction in the operation of the above-mentioned 1) were combined and diluted with 10 mM phosphoric acid buffer solution (pH 6.0) to 4 times. The solution was loaded onto the above-mentioned column at a flow rate of the 3 ml/min. The column was washed with 100 ml of 10 mM phosphoric acid buffer solutions (pH 6.0) after the sample application. The elution was carried out by the 60 minutes gradient in 0 to 0.7 M NaCl concentration (in 10 mM phosphoric acid buffer solution: pH 6.0). Hemolytic activity was showed in many fractions eluting between 45 and 65 minutes after start of the gradient. In addition, hemolytic activity was examined about the hemolytic effect to sheep hemocytes (see the after-mentioned example 2).

3) The purification by ion exchange HPLC (column: TSK-GEL CM5PW, column size: 7.5 x 75 mm)

The above-mentioned column was well equilibrated with 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.3 M NaCl. The hemolytic active fractions obtained by purifying operation of the above-mentioned 2) were diluted with 10 mM

phosphoric acid buffer solution (pH 6.0) to 4 times. The solution was loaded onto the above-mentioned column at the flow rate of 2 ml/min. The column was washed with 30 ml of 10 mM phosphoric acid buffer solutions (pH 6.0) after the sample application. After washing, the elution was performed by the 60 min gradient in 0 to 0.8 M NaCl concentration (in 10 mM phosphoric acid buffer solution: pH 6.0). Fractions having hemolytic activity were eluted between 25 and 35 minutes after start of the gradient, and each fraction was applied to SDS-PAGE. The separating condition of the active component was verified, and the portions separated well were collected and used in the next step. On the contrary, the portions not separated were further performed by chromatography to complete the separation of the active component.

4) Concentration of the hemolytic active component by ion exchange HPLC (column: TSK-GEL CM5PW, column size: 7.5 x 75 mm)

The column was well equilibrated with 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.3 M NaCl. The hemolytic active fractions obtained by purifying operation of above-mentioned 3) were diluted with 10 mM phosphoric acid buffer solution (pH 6.0) to 4 times. The solution was loaded onto the above-mentioned column at the flow rate of 2 ml/min. The column was washed with 30 ml of 10 mM phosphoric acid buffer solutions (pH 6.0) after the sample application. After washing, 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.8 M NaCl was then rinsed and the sample adhered into the column was allowed to elute. In about 5 minutes after exchange of

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the solvent, the portion of the hemolytic active component condensed and eluted at a stretch was collected.

5) The purification by gel filtration HPLC (column: Superdex-75, column size: 16 x 600 mm)

Every 0.5-1.0 ml of the sample condensed by ion exchange HPLC was applied to the above-mentioned column equilibrated with 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.8 M NaCl, and allowed to elute at the flow rate of 1 ml/min. Potent hemolytic activity was found out in the fraction eluting between 50 and 60 minutes after injection of the sample. After confirming the separating condition by SDS PAGE, the protein of the present invention, a hemolytic toxin, was separated by collecting the active fractions (about 1 μg).

Example 2: Measurement of the hemolytic activity

Measurement of the hemolytic activity in each purification step in the above-mentioned Example 1 and measurement of the hemolytic activity of the protein of the present invention finally obtained were performed as follows.

1) Method

Hemolytic activity was measured by hemolysis to a sheep erythrocyte. That is, every 200 μl of PBS(+) buffer solution containing 0.8% of sheep erythrocyte was put into the microwell plates of 96 wells (round bottom type). 10 μl of the solution dissolved the fraction obtained in each purification step of the above-mentioned Example 1 in 10 mM phosphoric acid buffer solution (pH 6.0) was added to the plate. It was allowed to stand at room temperature for 3 hours, and the hemolytic

condition of the sheep erythrocyte of each plate was observed. In addition, the presence or absence of the retention of the hemolytic activity was determined by whether the fraction obtained in each purification step exhibits a perfect hemolysis.

2) Results

2-1) The fraction obtained in each purification step of the above-mentioned Example 1 exhibited the perfect hemolysis to the sheep erythrocyte, and therefore, it became clear that it retains the hemolytic activity.

2-2) Moreover, the protein of the present invention having the hemolytic activity finally obtained by purification operation of the above-mentioned 5) in Example 1 caused the perfect hemolysis to the sheep erythrocyte in the concentration below 100 ng/ml (about 2 nM).

Example 3: Determination of the molecular weight and the partial structure on the proteins

3-1) Determination of the molecular weight

The single band visualized by applying the protein of the present invention having the hemolytic activity obtained by purification operation of 5) in Example 1 to SDS gel electrophoresis (SDS-PAGE) according to the conventional method was compared with the protein molecular-weight marker (Pharmacia Co.). As the result, it was identified that the molecular weight of the protein of the present invention are about 50,000 Da.

3-2) Decomposition with the lysylendopeptidase

The protein was decomposed by adding 3 pM of Achromobacter Protease I (derived from *Achromobacter lyticus* M497-1: Takara Shuzo Co.) to 10 μg of protein according to the present invention having the hemolytic activity obtained by purification operation of the above-mentioned 5) in Example 1, and incubating in 10 mM of Tris-HCl buffer solution (pH 9.0) at 30℃ for 20 hours. The protein digested with the enzyme was applied to the high performance liquid chromatography (column: Bakerbond wide pore ODS), and separated with the 60 min gradient in 10 to 62% of acetonitrile concentration (in water containing 0.1% of trifluoroacetic acid) at the flow rate of 0.7 ml/min. Consequently, three peptide fragments eluting respectively at a retention time 19, 23 and 27 minutes were obtained.

3-3) Determination of the amino acid sequence of each fragments by the amino acid sequencer

The amino acid sequence of three peptide fragments obtained as mentioned above was determined according to the conventional method using Shimadzu PSQ-1 protein sequencer (Shimadzu Co.).

As the result, three fragments have the following amino acid sequences (1) - (3), respectively:

Amino acid sequence (1):

Gly-Glu-Ile-Gln-Thr-Lys-Pro-Asp-Arg-Val-Gly-Gln-Ala-Thr (SEQ ID NO: 1)

Amino acid sequence (2):

Gly-Asn-Ala-Glu-His-Val-Ala-Ser-Ala-Val-Glu-Asn-Ala-Asn-

a

Arg-Val-Asn-Lys (SEQ ID NO:2)

Amino acid sequence (3):

a

Met-Ser-Asp-Gly-Phe-Tyr-Thr-Met-Glu-Asn-Ser-Asp-Arg-Arg-Lys (SEQ ID NO:3)

(wherein, an amino acid residue is written by the 3 letters notation defined by IUPAC and IUB).

The homology search about each fragment with which the amino acid sequence was determined as mentioned above exhibited that the homology between these fragments and the known proteins was very low. Therefore, it was suggested that the specific protein of the present invention fractionated from the nematocyst of *Carybdea rastonii* while retaining the hemolytic activity is completely novel protein.

Example 4: Determination of the full amino acid sequence of the protein and the gene encoding the amino acids

4-1) Preparation of total RNA of *Carybdea rastonii*

The tentacle (about 0.5 g in wet weights) of *Carybdea rastonii* was crushed in the liquid nitrogen, and homogenized in 5 ml TRIzol (registered trademark) reagent (GIBCO BRL Co.). To this mixture was added 1 ml of chloroform, and the mixture was agitated, and centrifuged with the cooling centrifuge (Sakuma Co.) [13,000rpm, for 15 minutes, at 4℃]. The upper aqueous layer was fractionated, and to this solution was added 2.5 ml of isopropanol, then, the mixture was allowed to stand at room temperature for 10 minutes. The supernatant fluid was

removed after the centrifugal separation (13,000rpm, for 10 minutes, at 4℃) using the cooling centrifuge, and then 5 ml of 75% ethanol was added the residue. The supernatant fluid was removed after the centrifuge (10,000rpm, for 5 minutes, at 4℃) to obtain the residue, then, the air-drying of the residue was performed for about 10 minutes. 100 µl of RNase-free water was added to the resulting residue, and the mixture was incubated for 10 minutes at 60℃ to lyse RNA. About 0.5 mg of total RNA was obtained according to the above-mentioned method.

4-2) Cloning of a partial cDNA

On the basis of amino acid sequence (1), amino acid sequence (2) and amino acid sequence (3), the following degenerate primers were designed and synthesized by the conventional method:

7-F; GAR ATH CAR ACI AAR CCI G (SEQ ID NO:6)

7-R; CIG GYT TIG TYT GDA TYT C (SEQ ID NO:7)

12-F; GCI GTI GAR AAY GCI AAY MG (SEQ ID NO:8)

12-R; CKR TTI GCR TTY TCI ACI GC (SEQ ID NO:9)

14-1-F; GAY GGI TTY TAY ACI ATG G (SEQ ID NO:10)

14-1-R; CCA TIG TRT ARA AIC CRT C (SEQ ID NO:11)

12-2-F; GAY GGI TTY TAY ACI ATG GAR AA (SEQ ID NO:12)

12-2-R; TTY TCC ATI GTR TAR AAI CCR TC (SEQ ID NO:13)

(wherein, the above-mentioned alphabetic character was written based on the "Nucleotide Abbreviation List" (Cell Technology, separate volume, "*Biotechnology Experiment Illustrated*": Shujunsha Co.).

Next, according to the following procedure, single-strand cDNA was synthesized using SUPERSCRIPT (registered trademark) Preamplification System for 1st-Strand cDNA Synthesis. That

is, 1 μg of total RNA , oligo(dT)$_{12-18}$, and DEPC-treated water were mixed, and the mixture was allowed to stand for 10 minutes at 70℃. Then, PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix, and 0.1 M DTT were added to this mixture, and the resulting mixture was pre-incubated for 5 minutes at 42℃. Superscript II RT (200 units/μl) was added to this mixture, and the mixture was incubated for 50 minutes at 42℃ and for 15 minutes at 70℃. The RNase H was added to the mixture, and then, the resulting mixture was incubated for 20 minutes at 37℃ to obtain 1st-strand cDNA.

Subsequently, according to the following conditions, PCR was performed using GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer Co.). That is, 1st-strand cDNA, PCR buffer, dNTP mix, primer 1 and primer 2 (wherein, primer 1 and primer 2 are any eight above-mentioned primers.), TaKaRa Ex Taq (registered trademark, Takara Shuzo Co.), and water were mixed. The reaction was performed by heating the mixture at 94℃ for 5 minutes and repeating 3 cycles of 30 seconds at 94℃, 30 seconds at 45℃ and 2 minutes at 72℃, and 27 cycles of 30 seconds at 94℃, 30 seconds at 55℃ and 2 minutes at 72℃. The reactant was then treated for 5 minutes at 72℃.

The obtained reaction solution was electrophoresed on 0.8% agarose gel to confirm the amplified PCR products in the combination of 7-F and 12-R, 7-F and 14-1-R, 7-F and 14-2-R, 12-F and 14-1-R, and 12-F and 14-2-R. The sizes of each PCR product were about 600bp, 1,000bp, 1,000bp, 400bp, and 400bp, respectively.

4-3) Sequencing of the partial cDNA

Each PCR product was inserted into TA cloning vector pCR2.1

(Invitrogene Co.), and the recombinant was transformed to the *Escherichia coli* JM109. The transformant was cultured on LB (containing 50 μg/μl of ampicillin) agar medium. According to the following conditions, colony PCR was performed to the colonies obtained as a template using the M13 universal primer. The strain of *Escherichia coli*, PCR buffer, dNTP mix, M13 FW primer, M13 RV primer, TaKaRa Ex Taq (registered trademark, Takara Shuzo Co.), and water were mixed. The reaction was performed by heating the mixture at 90℃ for 10 minutes and repeating 30 cycles of 30 seconds at 94℃, 30 seconds at 55℃ and 2 minutes at 72℃, and then heating at 72℃ for 5 minutes. The reaction solution was electrophoresed on 0.8% agarose gel and the target colony PCR product was purified on the spin column of MicroSpin (registered trademark) S-400 (Amersham Pharmacia Co.). Then, the sequencing of the obtained product was conducted using ABI PRISM 310 Genetic Analyzer (Applied Biosystems Co.).

The obtained sequence was analyzed using gene analysis software GENETYX-MAC (Software Development Co.). As the result, the partial cDNA sequence of about 1000 bp was analyzed, and each partial structure of amino acid sequence (1), amino acid sequence (2) and amino acid sequence (3) was determined to locate in this turn from N terminal of the protein.

4-4) Sequencing of the full-length cDNA

Following primers were synthesized based on the base sequence of the partial cDNA:

5'-RACE-4R; GCT CTA TCA ATA ACG GCA GC (SEQ ID NO:14)

5'-RACE-5R; TGT CTT TGG ATG GCC TCA TC (SEQ ID NO:15)

5'-RACE-6R; GAT ACT TAG GTC GCT ATC CG (SEQ ID NO:16)

3'-RACE-1F; GTT CAG AGG CTG TTC TAA CG (SEQ ID NO:17)

3'-RACE-2F; ATG TCT GAC GGC TTC TAC AC (SEQ ID NO:18)

Next, according to the following procedure, 5' RACE and 3' RACE were performed using 5'/3' RACE Kit (Boehringer Mannheim Co.).

(a) 5' RACE

1 μg of total RNA, cDNA synthesis buffer, dNTP mix, 5'-RACE-6R, AMV reverse transcriptase, and DEPC-treated water were mixed, and the mixture was incubated for 60 minutes at 55℃ and for 10 minutes at 65℃ to obtain 1st-strand cDNA.

Next, 1st-strand cDNA thus obtained was purified on the spin column, then, reaction buffer and 2mM dATP were added to the 1st-strand cDNA, and the mixture was allowed to stand for 3 minutes at 94℃. Terminal transferase (10 units/μl) was added to the mixture, and the resulting mixture was incubated for 20 minutes at 37℃. After the incubation, 1st-strand cDNA, PCR buffer, dNTP mix, 5'-RACE-5R, oligo(dT)-anchor primer, and water were added to the above mixture. The reaction was performed by heating the mixture at 94℃ for 5 minutes and repeating 30 cycles of 30 seconds at 94℃, 30 seconds at 55℃ and 1 minute at 72℃, and then heating at 72℃ for 5 minutes. Consequently, the nested-PCR was performed to the 1st-PCR product as a template using the combination of 5'-RACE-4R and PCR anchor primer under the same condition as 1st-PCR.

The 1st-PCR product and the nested-PCR product were electrophoresed on 1.5% agarose gel to confirm the band of about 500bp. This nested-PCR product was inserted into TA cloning vector, and the sequencing was performed according to the

determination of the base sequence of cDNA described in the above-mentioned 4-3), then the sequence was analyzed.

(b) 3' RACE

1 μg of total RNA, cDNA synthesis buffer, dNTP mix, oligo(dT)-anchor primer, AMV reverse transcriptase, and DEPC-treated water were mixed, and the mixture was incubated for 60 minutes at 55℃. Subsequently, the reactant was treated for 10 minutes at 65℃ to obtain 1st-strand cDNA.

Next, 1st-PCR thus obtained was performed under the following condition. 1st-strand cDNA, PCR buffer, dNTP mix, 3'-RACE-1F, PCR anchor primer, TaKaRa Ex Taq (registered trademark, Takara Shuzo Co.), and water were mixed. The reaction was performed by heating the mixture at 94℃ for 5 minutes and repeating 30 cycles of 30 seconds at 94℃, 30 seconds at 55℃ and 2 minutes at 72℃, and then heating at 72℃ for 5 minutes. The nested-PCR was performed to the 1st-PCR product as a template using the combination of 3'-RACE-2F and PCR anchor primer under the same condition as 1st-PCR.

The 1st-PCR product and the nested-PCR product were electrophoresed on 1.5% agarose gel to confirm the band of about 600 bp. The nested-PCR product was inserted into TA cloning vector, the sequencing was performed according to the determination of the base sequence of cDNA described in the above-mentioned 4-3), and the sequence was analyzed.

As a result, the size (1610bp) and the sequence of cDNA encoding the novel hemolytic active protein of *Carybdea rastonii*, and the number (450aa) and the sequence of amino acid of the protein became clear. That is, the hemolytic active protein

of *Carybdea rastonii* had the amino acid sequence represented by ~~SEQ ID NO 5~~ (SEQ ID NO:5), and the gene encoding thereof had the base sequence represented by ~~SEQ ID NO 4~~ (SEQ ID NO:4).

The amino acid sequence (1) ~~(SEQ ID NO 1)~~ (SEQ ID NO:1), the amino acid sequence (2) ~~(SEQ ID NO 2)~~ (SEQ ID NO:2), and the amino acid sequence (3) ~~(SEQ ID NO 3)~~ (SEQ ID NO:3) corresponded to the amino acid number 56-69 of ~~SEQ ID NO 5~~ (SEQ ID NO:5), the amino acid number 250-267 of ~~SEQ ID NO 5~~ (SEQ ID NO:5), and the amino acid number 363-377 of ~~SEQ ID NO 5~~ (SEQ ID NO:5), respectively. Furthermore, it was confirmed that the poly A sequence exists after the nucleotide number 1600 of ~~SEQ ID NO 4~~ (SEQ ID NO:4).

The novel protein of the present invention obtained as mentioned above is the specific protein having the following physiological activity, and physical and chemical property, as indicated by the example:

(a) having hemolytic activity;

(b) having a molecular weight of about 50,000 Da (determined by SDS gel electrophoresis);

(c) having the amino acid sequences 1 to 3 described above as a partial amino acid sequence; and

(d) having the amino acid sequence represented by SEQ ID NO 5 as the full amino acid sequence.

Industrial applicability

Since the protein having the hemolytic activity derived from the nematocyst of *Carybdea rastonii* provided according to the present invention is a novel protein which is not similar to known protein, as a result of the homology search on the partial amino acid sequence and the full primary amino acid

sequences, it is useful as a biochemical reagent for example, elucidating the mechanism of a hemolysis etc.

It also provides the new approach directed to development of drugs, such as the medicine for treating the sting by the jellyfish, on the basis of study of correlation of the structural activity in a molecular level, and the antibody on the protein or the partial peptide, etc. Furthermore, it is useful as the drugs having a platelet agglutination effect etc., and pesticides using a hemolytic activity.